IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

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| Applicant: | John F. Engelhardt et al. | Examiner: Kevin Hill |
| Serial No.: | 10/815,262 | Group Art Unit: 1633 |
| Filed: | March 31, 2004 | Docket No.: 875.074US1 |
| Customer No.: | 21186 | Confirmation No.: 7471 |
| Title: | COMPOUNDS AND METHODS TO ENHANCE rAAV TRANSDUCTION | |

## RULE 132 DECLARATION

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

We, Dr. John Engelhardt and Dr. Ziying Yan, declare and say as follows:

1. We are two of the co-inventors of the above-identified application and we make this Declaration in support of the patentability of the pending claims.

2. In the Office Action mailed December 4, 2008 for the above-referenced matter, the Examiner rejected claims 1-2, 4-7, 9-23, 43-44, 46, 48-50, 61, and 63-64 under 35 U.S.C. § 103(a) as being unpatentable over Duan et al. (J. Clin Invest., 105:1573 (2000)) in view of Kiyomiya et al. (Cancer Res., 61:2467 (2001)), Maitra et al. (Am. J. Physiol. Cell Physiol., 280:C1031 (2001)) and Engelhardt (U.S. Patent No. 6,436,392); claim 62 under 35 U.S.C. § 103(a) as being unpatentable over Duan et al. in view of Kiyomiya et al., Maitra et al. and Engelhardt et al. and further in view of Voinea et al. (J. Cell. Mol. Med., 6:465 (2002)); and claim 24 under 35 U.S.C. § 103(a) as being unpatentable over Duan et al. in view of Kiyomiya et al., Maitra et al., and Engelhardt et al. and further in view of Hirsch et al. (U.S. Patent Application Publication No. 2003/0003583).

3. Duan et al. disclose that the enhancement of AAV transduction by the combination of LLnL and EGTA might be due to reduced degradation of internalized virus and an increased rate of endocytosis. In this regard, the former activity can be attributed to LLnL, a proteasome inhibitor which does not alter AAV binding to cell surfaces or internalization, while

the latter activity can be attributed to EGTA, as it is not an inhibitor of proteasome proteolytic activity in AAV infected cells.

4. Kiyomiya et al. observed the nuclear transport of doxorubicin *in vitro* and Maitra et al. report on the effect of doxorubicin on total cellular CFTR protein expression, surface CFTR protein expression and CFTR-associated chloride secretion in T84 epithelial cells, and mutant CFTR cell surface expression and chloride secretion in stably transfected MDCK cells.

5. Neither Kiyomiya et al. nor Maitra et al. relate to AAV, and the '392 patent does not relate to agents that enhance AAV transduction.

6. The Examiner asserts that it would be obvious to combine proteasomal protease inhibitors, such as doxorubicin and LLnL, to achieve their expected results for their common purpose and that varying concentrations thereof, as part of routine optimization, would result in additive or synergistic enhancement of transduction.

7. Doxorubicin binds DNA, inhibits topoisomerase II, and is used to treat cancer. Prior to the present application, because of its associated cytotoxicity, doxorubicin would likely not be selected to treat patients without an imminently life-threatening disease. For example, as noted in Maitra et al., the use of doxorubicin in a clinical setting, e.g., to treat cystic fibrosis via altering CFTR expression, is counter indicated due to its cumulative systemic toxicity. In addition, the use of a toxic agent, such as doxorubicin, may compromise cell viability, thereby defeating the goal of enhanced transduction of cells with rAAV, e.g., a rAAV encoding a therapeutic gene product. Further, with regard to AAV, given that doxorubicin binds DNA, it might be expected to inhibit AAV transduction, as AAV is present as a double stranded DNA molecule in the nucleus.

8. In the absence of data, it is not predictable what combinations of agents have any effect, let alone have an additive or synergistic effect, on AAV transduction. For example, as shown in Figure 4 in Yan et al. (J. Virol., 78:2863 (2004)) (a copy is attached hereto),

combinations of LLnL or Z-LLL and doxorubicin resulted in a synergistic effect which would not occur if the two compounds acted through similar mechanisms. Compounds with similar mechanisms of action may, under some conditions, yield a positive, but not a synergistic, effect. However, the use of twice the amount of an agent (it has the "same" target) does not necessarily result in twice the effect (see, e.g., Figure 1 in Yan et al.).

9. As further support for the position that it is not predictable what combinations of agents have any effect, let alone a positive or synergistic effect, on AAV transduction, the Examiner is requested to consider the data attached hereto. The vast majority of the combinations tested resulted in an effect (on fold transduction) that was greater than additive, however, that effect could be further grouped into two categories: an increase in transduction for the combination that was greater than the sum of the increase in transduction for each individual agent but less than three times the sum (referred to below as a positive effect and noted as "P" in the Tables) or an increase in transduction for the combination that was greater than three times the sum of the increase in transduction for each individual agent (referred to below as a synergistic effect and noted as "S" in the Tables).

10. IB3, A549 and HeLa cells were infected with rAAV2 or rAAV5 and contacted with individual agents or combinations of agents, e.g., doxorubicin (selectively inhibits chymotrypsin-like proteolytic activity through binding to the subunits of 20S proteasomes), Velcade® (a chemotherapeutic that is a reversible inhibitor of the 26S mammalian proteasome), and/or LLnL (inhibits chymotryptic-, tryptic- and peptidylglutamyl peptide hydrolase-activities of the proteasome), and the induction fold measured (see the Tables and attached graphs).

11. For rAAV-2 and rAAV-5, a synergistic effect was observed in infected IB3 and A549 cells with a combination of doxorubicin (0.5 μM) and LLnL (10 μM). No synergistic or positive effect was observed for rAAV-2 or rAAV-5 infected IB3 cells treated with Velcade® (0.5 μM) and LLnL (10 μM), however, a positive effect was observed for rAAV-2 infected A549 cells treated with same concentrations of Velcade® and LLnL. No synergistic or positive effect was observed for rAAV-5 infected A549 cells with the same treatment. In addition, when IB3

and A549 cells were treated with Velcade® (0.5 μM) and doxorubicin (0.5 μM), a positive effect on AAV-2 transduction was only observed in IB3 cells; no synergistic or positive effect was observed for rAAV-2 infected A549 cells. When the cells were treated with the same concentrations of Velcade® and doxorubicin, a positive effect on AAV-5 transduction was observed in both A549 cells and IB3 cells. Table 1 below summarizes the fold induction observed for A549 and IB3 cells treated with a selected concentration of individual agents or treated with a combination thereof.

Table 1

| | IB3 | IB3 | A549 | A549 |
|---|---|---|---|---|
| Virus/treatment | AV2 | AV5 | AV2 | AV5 |
| Dox 0.5 μM | 10.3 | 23.4 | 3.1 | 2.6 |
| LLnL 10 μM | 6.5 | 7.4 | 6.3 | 1.7 |
| D+L | 42.7 | 66.8 | 53.2 | 24.6 |
| EFFECT | (P) | (P) | (S) | (S) |
| | | | | |
| Vel 0.5 μM | 52.3 | 34.9 | 32 | 10.9 |
| LLnL 10 μM | 6.5 | 7.4 | 6.3 | 1.7 |
| V+L | 55.3 | 34.5 | 71 | 13.4 |
| EFFECT | (N) | (N) | (P) | (N) |
| | | | | |
| Dox 0.5 μM | 10.3 | 23.4 | 3.1 | 2.6 |
| Vel 0.5 μM | 52.3 | 34.9 | 32 | 10.9 |
| D+V | 100 | 104 | 24.8 | 37.2 |
| EFFECT | (P) | (P) | (N) | (P) |
| | | | | |
| S=synergist | | | | |
| P=positive | | | | |
| N = none | | | | |

12. rAAV-2 infected HeLa cells treated with a combination of doxorubicin (1.0 μM) and Velcade® (1.0 μM) showed a negative effect on transduction, while rAAV-2 infected HeLa cells treated with a combination of doxorubicin (1.0 μM) and Velcade® (0.2 μM) showed a positive effect. rAAV-5 infected HeLa cells treated with a combination of doxorubicin (1.0 μM) and Velcade® (1.0 μM or 0.2 μM) showed a positive effect on transduction. Table 2 summarizes the HeLa cell data. Additional HeLa cell data is provided in the attachments.

Table 2

| | HeLa | |
|---|---|---|
| Virus/treatment | AAV2 | AAV5 |
| Dox 1 μM | 32.3 | 30.8 |
| Vel 0.2 μM | 44.1 | 9.45 |
| Dox 1 μM+Vel 0.2 μM | 111 | 62 |
| EFFECT | (P) | (P) |
| | | |
| Dox 1 μM | 32.3 | 30.8 |
| Vel 1 μM | 110 | 15 |
| Dox 1 μM+Vel 1.0 μM | 83 | 63 |
| EFFECT | (N) | (P) |

13. The enclosed data and Yan et al. demonstrate that agents that purportedly have the "same" target (the proteasome) can result in a synergistic effect on AAV transduction and that agents that have a common structure, e.g., anthracyclines or peptide-like molecules, alone or in combination, can enhance AAV transduction. Moreover, the enclosed data evidence that not all combinations of agents that individually enhance AAV transduction yield a positive or synergistic effect and that some combinations in fact reduce AAV transduction efficiency. Therefore, it is not predictable what combinations of agents have any effect, let alone a positive or synergistic effect, on AAV transduction.

14. The data also support the proposition that individual agents likely modulate proteasome activity and AAV transduction through their own unique mechanisms, e.g., there are two different pathways or steps in intracellular AAV processing related to proteasomes that may be altered and result in a synergistic effect on AAV transduction. When the specific mechanisms overlap, then at least a positive effect on AAV transduction may be observed. That may explain why some combinations of agents, e.g., doxorubicin + LLnL, showed more synergism than other combinations, for instance, doxorubicin + Velcade®, while other combinations showed no effect, e.g., Velcade® + LLnL, in IB3 and A549 cells.

15. For instance, proteasomes exist in the cytoplasm and the nucleus of cells, but may play different roles in controlling AAV transduction in those cellular compartments.

Proteasomes in the cytoplasm may control AAV transport to the nucleus, while proteasomes in the nucleus may control uncoating of the virion and release of the DNA into the nucleus. Thus, modulating both cytoplasmic and nuclear proteasomes in cell types where transduction of at least one serotype of AAV can be enhanced in both of those cellular compartments, may synergistically enhance AAV transduction.

16. The Examiner also asserts that because Maitra et al. disclose that the concentration of doxorubicin used *in vitro* (0.25 μM) was about 20-fold lower than the $LD_{50}$, Maitra et al. suggest that one might be able to achieve these effects *in vivo* at a low dose, suggesting further optimization to avoid toxicity yet retain desired activity.

17. Interestingly, the use of a 20-fold higher concentration of doxorubicin than that employed by Maitra et al. did enhance AAV transduction (see Figures 2 and 4 in Yan et al.). Moreover, higher concentrations of doxorubicin than that employed in Maitra et al. enhanced rAAV-2 and rAAV-5 transduction of IB3 cells (2 μM showed greater enhancement than 0.5 μM), A549 cells (5 μM showed greater enhancement than 1 μM) and HeLa cells (1 μM showed greater enhancement than 0.2 μM) (data not shown). Thus, the effect of certain concentrations of doxorubicin on CFTR protein expression or activity is not predictive of the effect of doxorubicin on AAV transduction, much less the effect of combinations of agents that include doxorubicin on AAV transduction.

18. We further declare that all statements made herein of our own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: May 5th, 2009 By: _____
Dr. John Engelhardt

Dated: May 5th, 2009 By: _____
Dr. Ziying Yan